ORIGINAL ARTICLE

Monitoring of Dengue Virus in Field-caught *Aedes* Species (Diptera: Culicidae) by Molecular Method, from 2016 to 2017 in Southern Iran

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Abstract

Background: *Aedes* mosquitoes transmit important arboviral diseases such as dengue to humans. This study was conducted to determine dengue virus infection in *Aedes* mosquitoes, emphasizing *Aedes aegypti* by Reverse Transcriptase-Polymerase Chain Reaction Assay from different regions in Southern Iran.

Methods: *Aedes* samples were collected by standard methods from different habitats of Hormozgan province, Southern Iran, in 2016-2017, and identified by morphological characteristics. In this study, TissueLyserII was used to homogenize the collected mosquitoes. In addition, the RT-PCR technique was used to identify dengue virus RNA.

Results: Overall, 1351 larval and adult *Aedes* mosquitoes were collected from five sites in Hormozgan Province, including 452 adults and 899 larvae. Five species from *Aedes* genera were collected (*Ae. aegypti, Ae. vittatus, Ae. caballus, Ae. caspius, Ae. vexans*). The investigations of dengue virus infection in *Aedes* mosquitoes showed no dengue virus infection in this species.

Conclusion: This study provides important information about *Aedes* mosquitoes. Vector control strategies must be emphasized and prioritized. Such actions prevent the establishment of *Aedes* mosquitoes and the spread of arboviral diseases in new areas. In addition, early detection of arboviruses in vectors and entomological monitoring can enhance the control measures for arbovirus diseases.

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Introduction

Mosquitoes have been considered the center of attention due to their important role in transmitting viral and parasitic diseases to humans and animals.[1,2] Most of the world's population live in high-risk areas where *Aedes* mosquitoes transmit various pathogens such as dengue and Zika viruses.[3] The World Health Organization (WHO) report in 2008 indicated that 247 million people became ill in 2006 and about one million died from mosquito-borne diseases.[4] Dengue fever is the most important arboviral disease transmitted by *Aedes* mosquitoes, a major international health issue. Outbreaks of dengue fever usually occur during the rainy and warm seasons. In the 1950s, the disease was reported in only nine countries, but today it is endemic in more than 100 countries. More than 40% of the world's population live in tropical and subtropical regions threatened by dengue fever. The annual number of new cases of this disease in the world is between 50-100 million, of which 400,000 cases experience the severe form of dengue, also known as Dengue hemorrhagic/shock syndrome. Around 15% of the patients with bleeding may die, among whom about 90% are children under 15 years old.[5,6] The causative agent of dengue fever is an RNA virus from genus Flavivirus in the family Flaviviridae.

Dengue fever is a disease caused by the mosquito-borne dengue viruses, including four serotypes (DENV 1 to 4).

Aedes aegypti and *Ae. Albopictus* are important vectors of this disease.[7] *Aedes* has aggressive and intermittent blood-eating behavior, which allows the transmission of multiple arboviruses to humans, so it is complicated to control arbovirus diseases.[8, 9] Vectors of dengue fever are widely distributed in tropical, subtropical, and temperate regions.[10, 11] Dengue fever and dengue hemorrhagic fever (DHF) outbreaks have been reported in many countries, including Indonesia, Thailand, Kenya, Mozambique, Djibouti, Somalia, Sudan, Saudi Arabia, Yemen, and Pakistan.[12, 13]

Climate change is the most critical factor in the mosquito-borne viruses such as dengue virus from subtropical regions to temperate regions in the world.[14] At the same time, the isolation of Dengue viruses (DENV) from patients is essential to monitor dengue fever. Additional information on mosquitoes, such as viral sequence, mosquito infection rate, and serotype/genotype replication (sequence), may help the researchers better understand the dynamics and rotation of dengue virus transmission. The virus can be monitored by collecting field mosquitoes for the presence and rotation of virus activity and monitoring control measures.[15, 16] However, the low rate of infection (usually about 0.1%) by DENV in adult female *Ae. aegypti* mosquitoes makes it difficult to detect them.[17] Asymptomatic people infected with DENV can transmit the virus to mosquitoes despite the low level of viremia. They may act as a hidden reservoir for mosquito infestation, which spreads DENV. In this scenario, the viral data of the mosquitoes collected in the field may indicate asymptomatic infections in humans.[18, 19] Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) is a valuable diagnostic tool with high sensitivity and specificity that can detect and identify the viral RNA. RT-PCR is more sensitive than virus isolation and can detect circulating serotypes, allowing the researchers to directly detect DENV-RNA in mosquitoes collected in the field.[20-23] By identifying infected *Ae. aegypti* mosquitoes and monitoring dengue patients, dengue epidemics can be predicted and prevented. The south of Iran is one of the high-risk areas where arbovirues are likely to be transmitted.[24] Therefore, the phonetic study of *Aedes* mosquitoes and their infection with dengue virus in these areas was performed using the RT-PCR method.

Methods

Study Area

Hormozgan province in Southern Iran is located at latitude 25º 24′–28º 57′ N and longitude 53º 41′–59º 15′ E. This province covers an area of approximately 68,379 km² and has a population of about 2 million people (Figure 1). This province has 13 cities and Bandar Abbas is its center. The average annual rainfall in this province is about 200 mm and the average humidity is 78%. In addition, the average maximum and minimum temperatures in this province are 52°C and 2°C, respectively (Figure 1).

Given the transmission possibility of the arbovirus vectors from the south of the country, suitable climatic conditions for mosquito growth, and possible disease occurrence, this study was performed in five cities of Bandar Abbas, Bandar Khamir, Bandar Jask, Bandar Lengeh, and Bashagard in Hormozgan province (Figure 1).

Collecting the Samples, Selecting the Study Sites, and Identifying the Samples

In the present study, in 2017, five locations with different habitats in five cities of Bandar Abbas, Bandar Khamir, Bandar Jask, Bandar Lengeh, and

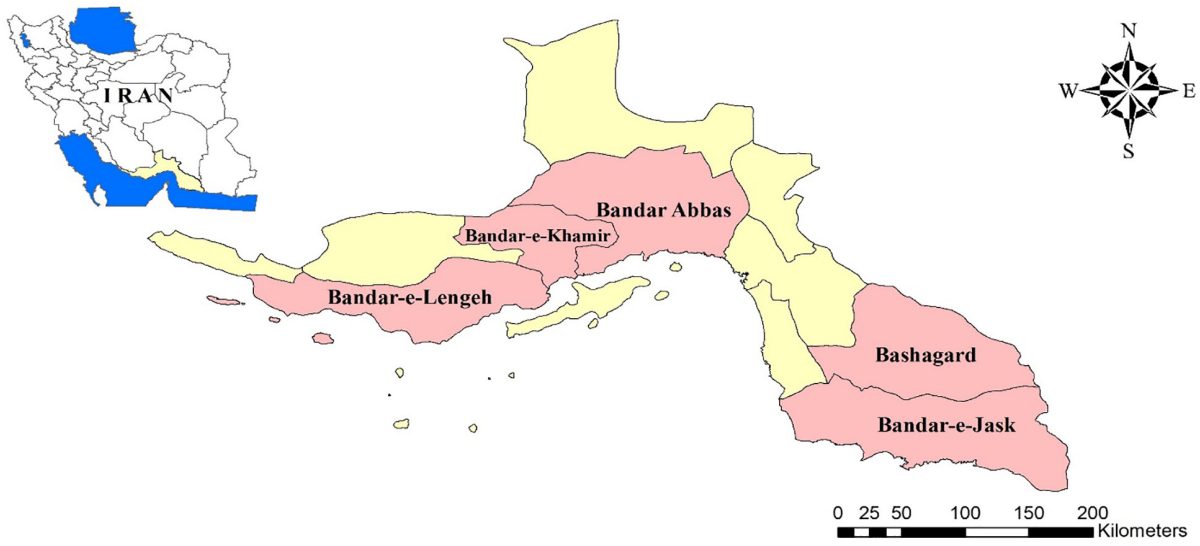


Figure 1: Study areas in Hormozgan province, Southern Iran (Photo source: Authors).

Bashagard from Hormozgan Province were selected to collect mosquitos, especially dengue fever vectors. Larval samples were collected using standard methods such as the dipping technique.[25] Adult mosquitoes were collected using various methods such as aspirators, mosquito nets, and light traps.[26] Larvae and adults of mosquitoes were identified using valid identification keys.[26]

After identification, the adult mosquitoes which were caught were transferred to the central laboratory by maintaining the cold chain in the nitrogen tank to determine possible infection with the dengue virus. After identifying the samples, 3-50 mosquitoes were placed inside a micro-tube. RNA Later solution was added to the samples and kept at -70 °C for analysis.

Molecular Assays for Detection of Dengue Virus

Firstly, the mosquitoes were homogenized using TissueLyserII (QIAGEN GmbH, Hilden, Germany); then, the homogenates were subjected to RNA extraction by the use of QIAamp® Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) based on the manufacturer's instruction. Finally, RT-PCR was done, using QIAGEN One-Step RT-PCR kit (QIAGEN GmbH, Hilden, Germany) as previously described (ref: An Imported Case of Dengue Fever in Iran, 2015).[27] Primers D1 (TCAATATGCTGAAACGCGCGAGAAACCG) and D2 (TTGCACCAACAGTCAATGTCTTCAGGTTC) were used to detect the dengue virus in *Aedes* mosquitoes.

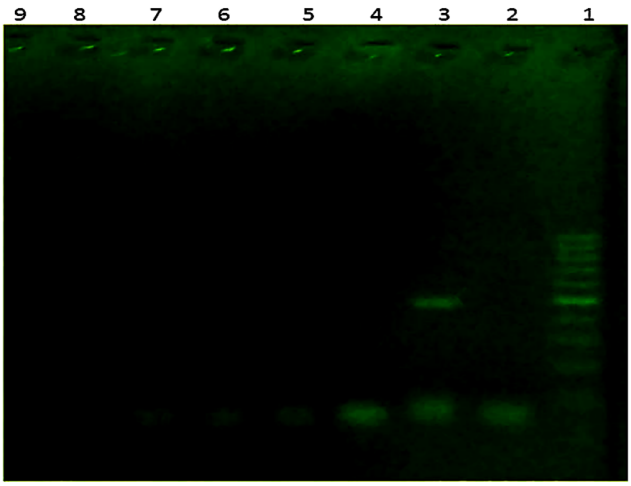
Results

In this study, 1351 mosquitoes were collected from 5 regions in Hormozgan province during 2017, including 452 adults and 899 larvae. Generally, five species of *Aedes* genera were identified, containing *Ae. aegypti, Ae. vittatus, Ae. caballus, Ae. caspius,* and *Ae. vexans* (Table 1). Adult specimens were caught from Human bait, animal bait, total catch, and hand catch indoor and outdoor. In the light trap method, no specimens of *Aedes* could be caught and the specimens were of Culex, which was not among the objectives of our study. In the light trap method, all the samples caught were Culex mosquitoes, which was not one of the objectives of our study. No Aedes was caught in the light trap method.

The most predominant species detected among the *Aedes* genus were adults and larvae of *Ae. vexans* species with 63.05% and 66.18%, respectively, in these areas (Table 1). To date, *Ae. aegypti* species were reported in cities, including Bandar-e Khamir and Bandar-e Lengeh, which were finally approved in the laboratory after identification by valid morphological keys.

Table 1: Characteristics and number of larvae and adults of *Aedes* species for determination of dengue virus infection by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) method, during 2017

Name of genus	Number of Larvae	Number of Adults	Cities									
			Bandar-e abbas (27°12'23.7"N 56°18'58.4"E)		Bandar-e khamir (26°57'01.9"N 55°34'56.3"E)		Bandar-e lengeh (26°32'44.4"N 54°52'45.0"E)		Bandar-e jask (25°38'58.0"N 57°46'53.9"E)		Bashagard (26°27'19.5"N 57°54'05.3"E)	
			Larvae	Adults	Larvae	Adults	Larvae	Adults	Larvae	Adults	Larvae	Adults
Ae. caspius	195	126	56	39	112	57	0	0	19	18	8	12
Ae. vexans	568	285	355	158	145	83	0	0	43	25	25	19
Ae. caballus	17	10	0	0	0	0	0	0	6	4	11	6
Ae. vittatus	72	23	0	0	72	23	0	0	0	0	0	0
Ae. aegypti	47	8	0	0	0	3	47	5	0	0	0	0
Total	899	452	411	197	329	166	47	5	68	47	44	37



1: Ladder 100bp
2: Negative Control
3: Positive Control
4-9: Samples

Figure 2: The negative result of the tests for possible infection with dengue virus in different species of *Aedes* mosquitoes by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Photo source: Authors)

Investigation of Possible Infection of Larvae and Adults Aedes Mosquitoes with Dengue Virus

Larvae and adult mosquitoes collected in Hormozgan province were tested for possible infection with dengue virus by RT-PCR method. The results of the molecular assays showed the specimens were not infected with dengue virus (Figure 2, Tables 2 and 3). Due to this point, the studied areas had suitable climatic conditions for the *Aedes* species development, so these areas are susceptible to the entry and endemicity of the dengue disease.

Discussion

The present study is the first research on the dengue virus in the field-caught *Aedes* mosquitoes by Reverse Transcriptase-Polymerase Chain Reaction Assay in southern Iran. Climatic conditions in Southern Iran, especially in Hormozgan province, are hot and humid, so these areas may become the ideal biological environment for mosquitoes to reproduce as disease vectors in the near future. Dengue is common in Pakistan and this country shares a border with Southern Iran, and many travelers come to Iran from this country. Moreover, *Ae. aegypti* and *Ae. albopictus* species have been introduced as the main vectors in this country.[28] As dengue fever has been reported in Iran, this study aimed to investigate the presence of the dengue virus in *Aedes* mosquitoes in Southern Iran.

Aedes mosquitoes are one of the most important vectors and transmit many important arbovirus diseases to humans.[29] In the present study, five species of the *Aedes* genus, such as *Ae. aegypti, Ae. vittatus, Ae. vexans, Ae. caballus,* and *Ae. caspius* were identified and reported in the study areas. Among the five species, *Ae. vexans* was the most abundant. *Ae. vexans* and *Ae. caspius* species have been reported from seven provinces in Iran, so the present study confirms the result of the previous study.[29]

The *Ae. aegypti* is an important vector of DENV in the world. Previous studies have shown that *Ae. aegypti* prefer indoor spaces to rest, while the present study showed that this species preferred both indoor and outdoor spaces.[30, 31]

One test on a young Iranian woman showed positive for IgM and RT-PCR after returning from India with symptoms of dengue virus (DENV)

Table 2: Results of larval infection of *Aedes* species with dengue virus by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) method, during 2017

Sampling Area	Name of genus	Larvae			
		Number of samples	Number of pools	Positive Pools	Dengue Viruses
Bandar abbas	Ae. caspius	56	2	0	-
Ae. vexans	355	9	0	-	
Bandar khamir	Ae. caspius	112	4	0	-
	Ae. vexans	145	6	0	-
	Ae. vittatus	72	2	0	-
Bandar lengeh	*Ae. aegypti*	47	2	0	-
Bandar jask	*Ae. caspius*	19	1	0	-
	Ae. vexans	43	2	0	-
	Ae. caballus	6	1	0	-
Bashagard	*Ae. caspius*	8	1	0	-
	Ae. vexans	25	1	0	-
	Ae. caballus	11	1	0	-

Table 3: Results of adult infection of *Aedes* species with dengue virus by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) method, during 2017

Sampling Area	Name of genus	Adult				
		Number of samples	Number of pools	Blood feeding	Positive Pools	Dengue Viruses
Bandar abbas	Ae. caspius	39	2	12	0	-
Ae. vexans	158	5	53	0	-	
Bandar khamir	Ae. caspius	57	2	16	0	-
	Ae. vexans	83	4	39	0	-
	Ae. vittatus	23	2	4	0	-
	Ae. aegypti	3	1	0	0	-
Bandar lengeh	Ae. aegypti	5	1	0	0	-
Bandar jask	*Ae. caspius*	18	2	5	0	-
	Ae. vexans	25	1	4	0	-
	Ae. caballus	4	1	0	0	-
Bashagard	*Ae. caspius*	12	1	2	0	-
	Ae. vexans	19	2	5	0	-
	Ae. caballus	6	1	0	0	-

infection.[32] A study was conducted on two Iranian patients with a history of travelling to Malaysia. The results of the genetic analysis showed that the dengue virus sequence in the phylogenetic tree specifically belonged to serotypes 1 and 3 of the dengue virus.[33] An Iranian researcher showed that a 52-year-old man from Hormozgan, three weeks after returning to Iran from Bahrain, had dengue fever symptoms confirmed by positive test result.[34] The results of the studies conducted in Iran show that all cases of the dengue virus disease have been imported, so the dengue virus transmission cycle has not yet been established in Iran. The results of our study also showed that Iranian *Aedes* mosquitoes were not yet infected with the dengue virus. This finding is consistent with the results of previous studies.

RT-PCR is one of the best and most practical diagnostic methods that is currently used in many laboratories. In most studies, this method is used to determine viral infection in mosquitoes. This diagnostic method helps to identify the dengue virus in larvae and pupae collected from the field. Many researchers have used this practical diagnostic technique in their studies to monitor DENV in *Aedes* mosquitoes, which act as a warning sign when dengue fever begins to spread and can predict the possible side effects of several DENV serotypes in circulation.[35, 36] Therefore, in this study, we also used the RT-PCR method to detect dengue virus infection, which showed negative results.

In Assam, a study was conducted using RT-PCR; The results showed that *Ae. albopictus* and *Ae. aegypti*, were the major vectors of dengue fever infected with the dengue virus.[37] In a study in Pakistan, Swat states measured the infection of vectors on various dengue fever serotypes using RT-PCR. The results showed that the vectors collected from around dengue patients' homes were infected with the dengue virus of the same strains.[38] Also, the results of a study in India showed that *Aedes* species were infected with the dengue virus when transmitting the eggs in the laboratory. Among the samples studied to determine the infection with dengue virus, the highest rate of infection was reported in *Ae.vittatus* at 20%.[39]

Currently, the vector control strategy in Iran is mostly focused on *Ae. aegypti*. However, few studies have been conducted to understand the role of other species in dengue fever transmission. In the present study, five types of *Aedes* species were tested by RT-PCR. The results showed that there was no dengue virus infection in Southern Iran. The difference between this investigation and other studies is that this disease has not been endemic in our country yet, but the main vectors of the dengue virus are present in the area. However, in other countries such as Pakistan, there are vectors and dengue patients, so all strategies must be used to prevent the establishment of dengue in Southern Iran.

Conclusion

The current study provides important information about *Aedes* mosquitoes. There are suitable environmental conditions for *Aedes* mosquitoes in Southern Iran. Arbovirus vectors such as *Ae. aegypti* are well adapted to different habitats in Southern Iran. It was found that the abundance of *Ae.aegypti* is currently low in the study areas. The priority of vector control strategies should be based on the elimination of mosquito larval reproduction sources. Such measures prevent the establishment of *Aedes* mosquitoes and the spread of arbovirus diseases in new areas of the region. In addition, this study showed the importance of arbovirus research in *Ae.aegypti* and other species. Early detection of arbovirus diseases in vectors along with entomological and virologic monitoring can enhance control measures for arbovirus diseases.

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Conflict of Interest: None declared.

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